Arent Fox
ATTORNEYS AT LAW

April 29, 2005

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Michael L. Mendelsohn
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
US Environmental Protection Agency
Document Processing Desk (7504C)
1801 S. Bell Street
Crystal Mall 2, Room 266A
Arlington, VA 22202

RE: 2004 Insect Monitoring Reports
EPA Reg. Nos. 524-489; 68467-2; 67979-1; 65268-1; and 29964-3

Dear Mr. Mendelsohn:

On behalf of the members of the Insect Resistance Management Technical Subcommittee (IRM Technical Subcommittee) of the Agricultural Biotechnology Stewardship Technical Committee, Arent Fox PLLC is submitting this letter and seven (7) copies of the enclosed monitoring reports. The IRM Subcommittee members hold the following registrations: Monsanto Company (EPA Reg. No. 524-489); Dow AgroSciences LLC (EPA Reg. No. 68467-2); Syngenta Seeds, Inc. – Field Crops-NAFTA (EPA Reg. No. 67979-1); Syngenta Seeds, Inc. – Vegetables – NAFTA (EPA Reg. No. 65268-1); and Pioneer Hi-Bred International, Inc. (EPA Reg. No. 29964-3). Neither the IRM Technical Subcommittee, nor any of its members, makes any claims of confidentiality with regard to this submission.

On October 15, 2001, the US Environmental Protection Agency (EPA) issued registration amendment letters to the *Bt* field corn registrants. These letters require, *inter alia*, the registrants to submit annually by April 30 a report on results of resistance monitoring and investigations of damage reports. This report is submitted in response to that requirement.

The enclosed submission contains the following third-party reports for Southwestern Corn Borer (SWCB), and Corn Earworm (CEW or *H. zea*) insect populations collected during the 2004 growing season:

- Monitoring the Susceptibility of the Southwestern Corn Borer, *Diatraea grandiosella*, to *Bacillus thuringiensis* Cry1Ab toxin. 2004. Q. Song, S. Wang and Y. Sun. University of Missouri.

- Monitoring the susceptibility of the Southwestern Corn Borer, *Diatraea grandiosella*, to *Bacillus thuringiensis* Cry1F protein. 2004. Q. Song, S. Wang and Y. Sun. University of Missouri.

- Monitoring Bt susceptibility of *H. zea* to Cry1Ab. 2004. Custom Bio-Products.

- Monitoring Bt susceptibility of *H.zea* to Cry1Fa. 2004. Custom Bio-Products.

A letter requesting an extension of time for the 2003/2004 European Cornborer monitoring reports was submitted to the Agency by each registrant. The submitted letters requested an extension to June 30, 2005 to enable the completion of bioassays on populations collected in 2004 as diapausing larvae.

Testing of the SWCB populations represents the continuation of the monitoring program to assess the susceptibility of field populations to the Cry1Ab and Cry1F proteins expressed in the registered corn products. The results of the dose response tests (i.e. LC_{50}, EC_{50}) were within the historical ranges and no significant changes were observed compared to previous years. No resistance alleles were detected using the diagnostic concentrations.

For the CEW program, insect populations were collected and analyzed using dose-response regressions and diagnostic bioassays. No significant changes in dose response were seen when compared to previous years. The concentrations for the 2004 diagnostic dose bioassays were increased to meet the Agency's request for an LC_{99} level dose (40 ng/cm^2 increased to 80 ng/cm^2 for Cry1Ab and 7500 ng/cm^2 increased to 54675 ng/cm^2 for Cry1Fa). This resulted in 99%+ mortality for all populations tested in both cases.

The registrants received no legitimate reports of unacceptable product performance for control of the target pests; therefore, no reports of such investigations are included the summary report included as part of this submission and listed below:

- 2004 Summary of Reports of Unacceptable Product Performance Against Target Pests for EPA Reg. Nos. 524-489; 68467-2; 67979-1; 65268-1; and 29964-3

If the Agency has any technical questions or concerns with the reports or this submission, please immediately contact Dr. Elizabeth D. Owens, (IRM Technical Subcommittee Chair, tel. 515-270-4083 or via email at Elizabeth.Owens@pioneer.com.

Sincerely,

Stanley H. Abramson
Authorized Representative of the
Agricultural Biotechnology Stewardship
Technical Committee

Enclosures

Summary of Reports of Unacceptable Product Performance Against Target Pests for EPA Reg. Nos. 524-489; 68467-2; 67979-1; 65268-1; and 29964-3

2004

Requirement: On October 15, 2001, the US Environmental Protection Agency (EPA) issued registration amendment letters to the *Bt* field corn registrants. These letters require, *inter alia*, the registrants to submit annually by April 30 a report on results of resistance monitoring and investigations of damage reports. This report summarizing individual registrant results of investigations of crop damage attributed to target pests is submitted in response to that requirement.

Summary for 2004: Registrants received no legitimate reports of unacceptable product performance for control of the target pests.

MONITORING Bt SUSCEPTIBILITY OF *H. zea* TO CRY1Fa

2004 Collections and Assays

Custom Bio-Products

INTRODUCTION: Dose response bioassays were conducted with the Cry1Fa toxin from Bacillus thuringiensis (Bt)) toxin against 7 geographically distinct populations of corn earworm (*Helicoverpa zea*) which were collected across the U.S. cotton belt plus a laboratory reared colony maintained at the USDA-ARS laboratory in Stoneville, MS. In addition, diagnostic bioassays were conducted on 5 of the field collections in addition to the laboratory colony. The bioassays were conducted using the protocol developed at the University of Nebraska (Siegfried et al. 2000).

OBJECTIVE: Compare levels of susceptibility to Cry1Fa among geographically distinct *H. zea* populations using dose-response regressions and diagnostic bioassays.

METHODS:

Colony Acquisition and Rearing:

F1 *H. zea* eggs from field collected adults were obtained from Dr. Carlos Blanco at the USDA-ARS laboratory in Stoneville, MS. Dose response and/or diagnostic bioassays were conducted using neonates from these eggs unless it was necessary to rear an additional 1 to 2 generations to obtain an adequate supply of test insects for bioassays.

Populations were reared using standard rearing protocols appropriate for this species. Eggs were collected from mated females and allowed to hatch. Neonate larvae were then used for bioassays and/or to initiate another generation. If available, at least 256 neonate larvae per population were infested into 128 rearing wells to increase colony size. Bioassays were initiated or resumed once eggs became available.

2004 *H. zea* populations received for bioassay

Collection Site	Date(s) Obtained
Maricopa, AZ	June 30
College Station, TX	July 1, 8
Corpus Christi, TX	July 1, 8
Auburn, AL	Aug. 11
Quincy, FL	Sept. 10, 14
Stoneville, MS	Sept. 14
Lubbock, TX	Oct. 13
Laboratory (USDA Stoneville, MS)	Various as needed
Tipton, GA	Oct. 13
Gainesville, FL	Nov. 3
AR State colony #20	Dec. 2
AR State colony #37	Dec. 2

Bioassays:

Bioassay of neonate larvae involved exposure to Bt dilutions applied to the surface of single wells of artificial diet. The larvae used in bioassays were from adults that had been reared at least one generation in the lab. Bioassays were performed in 128 well trays (each well 16 mm diameter x 16 mm height; CD International, Pitman, NJ). Dilutions of Bt were prepared in 0.1% Triton-X 100 to obtain uniform spreading of Bt solution on the diet surface.

Individual neonate larvae (less than 24 h after hatching) were selected at random and placed in the treated wells. Mortality and surviving larval weight were recorded 7 days later. Control treatments consisted of wells treated with 0.1% Triton-X 100. When recording mortality, larvae that had not grown beyond 10.0 mg in weight were considered to be dead. Bioassays were conducted over time and across generations (if needed) to run 6 replications of dose response bioassays on every population and 3 replications of diagnostic bioassays on 5 of the wild populations plus the lab colony. Each 128 well tray consisted of one replication. Within each rep of the dose response bioassay were 8 levels of Bt exposure; 0 (control), 25, 75, 225, 675, 2025, 6075, and 18225 ng Bt protein/cm^2 of diet surface. Each dilution was applied to 16 individual wells. Each rep of the diagnostic bioassay consisted of 16 wells of 0.0 (control) and 112 wells of 54,675 ng Bt protein / square cm surface area of diet.

The protein used for bioassays consisted of truncated Cry1Fa provided by Dow AgroSciences (Indianapolis, IN; Lot# 1599-45; 137 mg Cry1Fa / g formulation).

RESULTS and DISCUSSION:

The final test data from the field collections were collected December 6, 2004.

The colonies collected from Auburn, AL, and #'s 20 and 37 from AR State did not produce viable eggs and subsequently these colonies died off before any bioassays could be conducted.

The enclosed tables will show analysis for EC50, EC95 & EC99's (TABLE 1) and LC50, LC90 & LC99's (TABLE 2). TABLE 3 lists % mortality data from diagnostic concentration bioassays.

REFERENCES:

Siegfried, B.D., T. Spencer, and J. Nearman. 2000. Baseline Susceptibility of the Corn Earworm (Lepidoptera: Noctuidae) to the Cry1Ab Toxin from *Bacillus thuringiensis*. Journal of Economic Entomology Vol. 93, no. 4.

LeOra Software. 1987. POLO-PC. A user's guide to probit and logit analysis. Berkeley, CA

SAS Institute Inc. 1988. SAS Procedures Guide, Release 6.03 Edition. Cary-NC SAS Institute Inc., 441p.

TABLE 1
EC Data

Susceptibility of Corn Ear Worm populations to Cry1Fa

CRY1A POPULATION	# OF REPS	EC_{50}(95% CL) NG A.I./CM^2	EC_{90}(95% CL) NG A.I./CM^2	EC_{99}(95% CL) NG A.I./CM^2
College Station, TX	6	**12.56** (9.95 – 15.07)	**387.28** (274.67 – 547.81)	**2647.42** (1327.68 – 4600.30)
Corpus Christi, TX	6	**59.18** (51.41 – 67.45)	**2201.73** (1462.53 – 3225.82)	**16720.08** (8174.70 – 28970.98)
Maricopa, AZ	6	**120.58** (79.19 – 174.63)	**12615.12** (3826.86 – 35548.69)	**171044.14** (17265.02 – 674831.01)
Laboratory (USDA Stoneville, MS)	6	**182.42** (151.35 – 218.69)	**8766.96** (5008.30 – 14509.08)	**76853.99** (29062.03 – 154377.79)
Lubbock, TX	6	**98.20** (83.78 – 114.16)	**5047.18** (3139.74 – 7820.75)	**45943.60** (20176.54 – 85530.07)
Tipton, GA	6	**51.41** (44.42 – 58.77)	**2286.81** (1512.74 – 3373.48)	**19195.28** (9267.02 – 33771.35)
Quincy, FL	6	**97.30** (76.59 – 121.04)	**12120.49** (6073.13 – 22869.52)	**181230.45** (52355.60 – 444038.27)
Gainesville, FL	6	**57.85** (50.39 – 65.75)	**2687.29** (1805.91 – 3906.06)	**23112.30** (11559.38 – 39802.94)

12.56 - 120.58

TABLE 2

LC Data

Susceptibility of Corn Ear Worm populations to Cry1Fa

CRY1A POPULATION	# OF REPS	LC_{50}(95% CL) NG A.I./CM^2	LC_{90}(95% CL) NG A.I./CM^2	LC_{99}(95% CL) NG A.I./CM^2	SLOPE	CHI^2 PROBABILITY
College Station, TX	6	**45.30** (12.93 – 90.53)	**496.78** (255.54 – 1532.07)	**3500.30** (1224.48 – 36544.03)	1.232 +- 0.140	0.868
Corpus Christi, TX	6	**496.77** (199.74 – 846.95)	**4027.97** (2408.25 – 9336.74)	**22187.03** (9515.50 – 127271.28)	1.410 +- 0.251	0.844
Maricopa, AZ	6	**2711.81**(2218.94 – 3207.61)	**6995.67** (5714.68 – 9310.71)	**15148.37** (11006.00 – 24922.68)	3.114 +- 0.164	0.067
Laboratory (USDA Stoneville, MS)	6	**1144.39** (620.13 – 1775.28)	**8077.60** (5197.41 – 14988.33)	**39737.43** (20072.40 – 125025.54)	1.510+- 0.125	0.939
Lubbock, TX	6	**2177.27** (851.60 – 3391.73)	**9167.79** (5725.36 – 28172.33)	**29598.83** (13593.13 – 315165.33)	2.053 +- 0.307	0.914
Tipton, GA	6	**833.81** (599.40 – 1084.27)	**5024.61** (3751.49 – 7420.81)	**21728.72** (13293.19 – 44809.49)	1.643+- 0.176	0.557
Quincy, FL	6	**2828.28** (1656.86 – 4134.36)	**13354.95** (9123.23 – 22903.08)	**47339.62** (26655.64 – 127169.27)	1.901 +- 0.166	0.894
Gainesville, FL	6	**739.63** (248.67 – 1325.77)	**5260.83** (2793.12 – 21015.57)	**26042.77** (9278.38 – 432450.50)	1.504+- 0.163	0.992

~~496.78~~ =

45.30 - 2828.28

TABLE 3

Mortality by colony exposed to Cry1Fa diagnostic concentration

Colony	# Reps	ng/cm sq	Total N	% Mortality
Maricopa, AZ	3	54675	336	99.7
College Station, TX	3	54675	336	100.0
Tipton, GA	3	54675	336	100.0
Quincy, FL	3	54675	336	99.1
Gainesville, FL	3	54675	336	100.0
Laboratory (USDA Stoneville)	3	54675	336	100.0

MONITORING Bt SUSCEPTIBILITY OF *H. zea* TO CRY1Ab

2004 Collections and Assays

Custom Bio-Products

INTRODUCTION: Dose and diagnostic bioassays were conducted with the Cry1Ab toxin from Bacillus thuringiensis (Bt) toxin against 7 geographically distinct populations of corn earworm (*Helicoverpa zea*) which were collected across the U.S. cotton belt plus a laboratory reared colony maintained at the USDA-ARS laboratory in Stoneville, MS. The bioassays were conducted using the protocol developed at the University of Nebraska (Siegfried et al. 2000).

OBJECTIVE: Compare levels of susceptibility to Cry1Ab among geographically distinct *H. zea* populations using dose-response regressions and diagnostic bioassays.

METHODS:

Colony Acquisition and Rearing:

F1 through F3 *H. zea* eggs and/or pupae from field collected adults were obtained from Dr. Carlos Blanco at the USDA-ARS laboratory in Stoneville, MS. Dose response and diagnostic bioassays were conducted using neonates from these collections unless it was necessary to rear an additional 1 to 2 generations to obtain an adequate supply of test insects for 6 replications of bioassays.

Populations were reared using standard rearing protocols appropriate for this species. Eggs were collected from mated females and allowed to hatch. Neonate larvae were then used for bioassays and/or to initiate another generation. If available, at least 256 neonate larvae per population were infested into 128 rearing wells to increase colony size. Bioassays were initiated or resumed once eggs became available.

2004 *H. zea* populations received for bioassay

Collection Site	Date(s) Obtained
Maricopa, AZ	June 30
College Station, TX	July 1, 8
Corpus Christi, TX	July 1, 8
Auburn, AL	Aug. 11
Quincy, FL	Sept. 10, 14
Stoneville, MS	Sept. 14
Lubbock, TX	Oct. 13
Laboratory (USDA Stoneville, MS)	Various as needed
Tifton, GA	Oct. 13
Gainesville, FL	Nov. 3
AR State colony #20	Dec. 2
AR State colony #37	Dec. 2

8 locations
2 AR colonies + 1 lab colony
10

AZ, AL, TX, MS, FL, GA, AR

Bioassays:

Bioassay of neonate larvae involved exposure to Bt dilutions applied to the surface of single wells of artificial diet. The larvae used in bioassays were from adults that had been reared at least one generation in the lab. Bioassays were performed in 128 well trays (each well 16 mm diameter x 16 mm height; CD International, Pitman, NJ). Dilutions of Bt were prepared in 0.1% Triton-X 100 to obtain uniform spreading of Bt solution on the diet surface.

Individual neonate larvae (less than 24 h after hatching) were selected at random and placed in the treated wells. Mortality and surviving larval weight were recorded 7 days later. Control treatments consisted of wells treated with 0.1% Triton-X 100. When recording mortality, larvae that had not grown beyond 10.0 mg in weight were considered to be dead. Bioassays were conducted over time and across generations (if needed) to run 6 reps per test per population. Each 128 well tray consisted of one replication. Within each rep of the dose response bioassay were 8 levels of Bt exposure; 0 (control), 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 ng Bt protein / cm^2 of diet surface. Each dilution was applied to 16 individual wells. Each rep of the diagnostic bioassay consisted of 16 wells of 0.0 (control) and 112 wells of 80.0 ng Bt protein / square cm surface area of diet.

The protein used for bioassays consisted of a formulated Cry1Ab provided by Dow AgroSciences (San Diego, CA; Lot# MR818 571-1457; 11.7 mg Cry1Ab / g formulation).

RESULTS and DISCUSSION:

The final test data from the field collections were collected December 13, 2004.

The colonies collected from Auburn, AL, and #'s 20 and 37 from AR State did not produce viable eggs and subsequently these colonies died off before any bioassays could be conducted.

The enclosed tables will show analysis for EC50, EC95 & EC99's (TABLE 1) and LC50, LC90 & LC99's (TABLE 2). TABLE 3 lists % mortality data from diagnostic concentration bioassays

REFERENCES:

Siegfried, B.D., T. Spencer, and J. Nearman. 2000. Baseline Susceptibility of the Corn Earworm (Lepidoptera: Noctuidae) to the Cry1Ab Toxin from *Bacillus thuringiensis*. Journal of Economic Entomology Vol. 93, no. 4.

LeOra Software. 1987. POLO-PC. A user's guide to probit and logit analysis. Berkeley, CA

SAS Institute Inc. 1988. SAS Procedures Guide, Release 6.03 Edition. Cary-NC SAS Institute Inc., 441p.

TABLE 1
EC Data

Susceptibility of Corn Ear Worm populations to Cry1Ab

CRY1A POPULATION	# OF REPS	EC_{50}(95% CL) NG A.I./CM^2	EC_{90}(95% CL) NG A.I./CM^2	EC_{99}(95% CL) NG A.I./CM^2
College Station, TX	6	**0.081** (0.056 – 0.107)	**1.046** (0.949 – 1.168)	**4.378** (3.215 – 5.884)
Maricopa, AZ	6	**0.278** (0.170 – 0.385)	**12.222** (6.527 – 23.187)	**101.849** (26.636– 265.659)
Laboratory (USDA Stoneville, MS)	6	**0.487** (0.393 – 0.578)	**15.673** (10.164 – 23.868)	**109.702** (47.704 – 205.976)
Lubbock, TX	5	**0.223** (0.102 – 0.341)	**5.283** (2.660 – 11.170)	**31.117** (6.498 – 92.248)
Tipton, GA	6	**0.108** (0.065 – 0.154)	**3.803** (2.773 – 5.410)	**27.980** (13.013– 53.327)
Corpus Christi, TX	6	**0.284** (0.239 – 0.326)	**3.528** (2.796 – 4.427)	**14.486** (9.021 – 21.316)
Quincy, FL	6	**0.108** (0.065 – 0.154)	**3.803** (2.773 – 5.410)	**27.980** (13.013 – 53.327)
Gainesville, FL	6	**0.133** (0.107 – 0.159)	**5.450** (4.502 – 6.654)	**43.756** (28.904 – 63.469)

0.081 – 0.284

Lubbock, TX colony had only 5 reps due to limited egg supply.

7 colonies

TABLE 2

LC Data

Susceptibility of Corn Ear Worm populations to Cry1Ab

CRY1A POPULATION	# OF REPS	LC_{50}(95% CL) NG A.I./CM^2	LC_{90}(95% CL) NG A.I./CM^2	LC_{99}(95% CL) NG A.I./CM^2	SLOPE	CHI^2 PROBABILITY
College Station, TX	6	**0.154** (0.023 – 0.358)	**2.960** (1.808 – 5.846)	**33.048** (12.935– 276.791)	0.997 +- 0.143	0.722
Maricopa, AZ	6	**6.705** (5.670 – 7.756)	**19.246** (16.467 – 23.206)	**45.462** (35.818 – 62.174)	2.799 +- 0.224	0.298
Laboratory (USDA Stoneville, MS)	6	**4.166** (2.971 – 5.439)	**19.513** (15.529 – 25.315)	**68.708** (49.051 – 108.115)	1.911 +- 0.137	0.614
Lubbock, TX	5	**2.730** (2.030– 3.390)	**7.894** (6.388 – 10.490)	**18.759** (13.440 – 31.892)	2.779 +- 0.367	0.092
Tipton, GA	6	**2.389** (1.333 – 3.528)	**11.224** (7.790 – 17.438)	**39.756** (24.039 – 91.407)	1.905 +- 0.160	0.939
Corpus Christi, TX	6	**2.146** (1.705 – 2.610)	**9.922** (7.985 – 12.994)	**34.567** (24.281 – 55.677)	1.927 +- 0.164	0.512
Quincy, FL	6	**8.927** (4.535 – 13.348)	**31.208** (22.098 – 47.118)	**86.578** (55.446 – 190.943)	2.358 +- 0.193	0.982
Gainesville, FL	6	**4.161** (2.420– 6.208)	**24.368** (16.820 – 38.637)	**102.942** (60.221 – 232.796)	1.670 +- 0.097	0.994

0.154 - 8.927

TABLE 3

Mortality by colony exposed to Cry1Ab diagnostic concentration

Colony	# Reps	ng/cm sq	Total N	% Mortality
Corpus Christi, TX	6	80	672	100.0
College Station, TX	6	80	672	100.0
Gainesville, FL	6	80	672	99.4
Laboratory (USDA Stoneville)	6	80	672	99.7
Maricopa, AZ	6	80	672	100.0
Lubbock, TX	5	80	560	100.0
Tipton, GA	6	80	672	100.0
Quincy, FL	6	80	672	99.6

Monitoring the susceptibility of the Southwestern Corn Borer, *Diatraea grandiosella*, to *Bacillus thuringiensis* Cry1F protein

2004 Data Summary

Qisheng Song, Songjie Wang and Yaning Sun

Department of Entomology, Division of Plant Sciences, University of Missouri
Columbia, MO 65211

(April 23, 2005)

INTRODUCTION

The effectiveness of the resistance management strategies is measured by monitoring field populations. This allows for early detection of resistance so that selected management strategies can be implemented before control failure occurs. To monitor the susceptibility of the southwestern corn borer (SWCB), *Diatraea grandiosella*, a serious pest in the southern United States, to *Bacillus thuringiensis* (*Bt*) insecticidal protein in transgenic maize, SWCB populations are collected from areas where the selection pressure for resistance is the greatest, i.e. the areas with the highest market penetration of Bt corn hybrids and/or the areas with the highest insecticide application.

In this report, seven field populations of the SWCB were collected from two regions with high *Bt*-corn penetration rate and assayed along with a lab colony to determine susceptibility (larval mortality, larval growth inhibition and response to diagnostic dose) to Cry1F insecticidal protein. The data were then compared with the previous years' data for any sign of changes in the susceptibility of SWCB to Cry1F insecticidal protein.

EXPERIMENT PROCEDURES

a. Field SWCB sample collection

The SWCB field samples were collected from the same/nearby locations with a

high *Bt*-corn penetration rate and/or a history of insecticide application in refuge areas, as we did in the previous years. Dr. Dan Moellenbeck, DM Crop Research Group, under contract to ABSTC, collected three populations of SWCB from region 2 (Southwest Kansas and the panhandle area of Oklahoma and Texas) and our laboratory collected four SWCB field populations from region 4 (MO, IL, TE, and KY).

In region 2, three populations of SWCB diapausing larvae were collected from Garden City (Finney County) (153 larvae), Cimarron (Gary County) (112) and Great Bend (Stafford County) (62 larvae) of KS by Dr. Moellenbeck. In region 4, a total four SWCB populations were collected. One population of second flight adults (103 females at Dexter, MO) and one population of third flight adults (73 females at Princeton, KY) were collected using a blacklight trap over two or three consecutive nights. Insufficient adults were collected from the second and third flights at other locations (<10 females/location), presumably due to low density and/or unfavorable weather conditions. As an alternative, we traveled in early October to the same areas and collected one population of diapausing larvae from Delta Center, MO (188 diapausing larvae) and one from Ullin, IL (203 diapausing larvae) (Table 1).

b. Rearing of SWCB

Larvae generated from the field-collected populations and from a Mississippi (MS) laboratory colony were maintained on a BioServe artificial diet in clear plastic cups (20 ml) using established laboratory procedures (Chippendale and Cassatt, 1985). Larvae were kept at 30^0C under 16 hr L : 8 hr D, whereas eggs, pupae, and adults were incubated at 25^0C under 13 hr L : 11 hr D.

c. Cry1F insecticidal protein

Cry1F insecticidal protein was provided by Dow AgroSciences (800 mg Cry1F microbial protein powder, batch #TSN101788) and kept at -80^0C until use.

d. Susceptibility (LC_{50} and EC_{50}) of SWCB neonate larvae to Cry1F insecticidal protein

Larval mortality

A larval feeding bioassay was used to determine mortality. All bioassays were performed in a 128-cavity tray using diet incorporated serial concentrations of Cry1F insecticidal protein. A minimum of 120 larvae were tested per concentration.

Cry1F insecticidal protein was diluted in a 50 ml centrifuge tube containing 1 ml of distilled water to make a serial dilution of desired concentrations. Each solution was incorporated into 40 ml of added artificial diet (kept at 55^0C in a water bath) by inverting and shaking the tube. One ml of diet containing the indicated concentration of Cry1F insecticidal protein was then pipetted into a 128-cavity tray (BIO BA 128 bioassay tray, C-D International, Ocean City, NJ). The trays were either assayed shortly after cool down to room temperature or kept at 4^0C for < 48 h before use. Newly hatched and actively moving larvae (<24 h) were transferred individually to each cavity containing 1 ml of diet with the indicated concentration of Cry1F insecticidal protein. Each concentration was replicated four times (32 larvae replicate^{-1}) using larvae from different batches of the F_1 or F_2 generations (if the number of F_1 larvae from field collected females were not large enough, F_2 larvae were used). Both F_2 and F_3 generation larvae were used for feeding bioassay if the field population was from diapausing larvae. Larval mortality was observed at 14 days after treatment (DAT). Larvae were considered dead if they did not move when they were probed using a camel hairbrush or if they remained in the first instar.

Seven consecutive concentrations of Cry1F protein, including the control, were used for probit analyses to determine LC_{50} and LC_{95} values on 14 DAT. These concentrations produce larval mortality between 0 and 100%. Probit analyses were conducted, using SAS program (SAS Institute Inc., Cary, NC).

Larval growth inhibition

Six concentrations of Cry1F were used in bioassay. Each concentration was replicated 4 times (32 larvae replicate^{-1}) from different batches as described above. The mean weight of newly hatch larvae (initial weight) was determined by weighing a group

of 32 larvae and the mean weight of those larvae surviving at each tested concentration was determined at 14 DAT.

Regression analyses (log concentration vs. weight gain inhibition [%]) were used to determine the EC_{50} and EC_{95} values at 14 DAT. Weight gain inhibition represents the relative differences in weight gain of the treated larvae compared to that of the control larvae. The following equation was used to correct for initial weight: $I = \{(C - T) \div (C - B)\} \times 100\%$, where I = weight gain inhibition (%); C = mean weight of control larva (mg $larva^{-1}$); T = mean weight of surviving larvae from the treated diets (mg $larva^{-1}$); and B = mean initial larval weight (mg $larva^{-1}$).

Efficacy of the diagnostic concentration against the laboratory-adapted and field-collected populations of Diatraea grandiosella

The diagnostic concentration was estimated from the LC_{99} value at 14 DAT of the MS Laboratory population (obtained in 2000 from Dr. Frank Davis at the USDA-ARS laboratory in Stoneville, MS) because this population has been found to be the least susceptible to Cry1F insecticidal protein of the populations tested . Seven field populations of SWCB were used to test the efficacy of the diagnostic concentration (68.6 µg/g diet for 14 DAT) in determining susceptibility to Cry1F.

Two hundred newly hatched larvae from the F_2 or F_3 generation of each field population were transferred individually into the 128-cavity tray containing 1 ml of control diet or the diet with the diagnostic concentration of Cry1F insecticidal protein. On 14 DAT, larval mortality was recorded.

Results and Discussions

Two bioassay procedures (larval mortality vs. growth inhibition) were employed to investigate the susceptibility of SWCB to Cry1F. Based on the LC_{50} (Table 2) and EC_{50} (Table 3), significant variation [LC_{50} from 0.87 to 2.57 µg/ml diet for 14 DAT (Table 2), EC_{50} from 0.072 to 0.104 µg/ml diet for 14 DAT (Table 3)] was observed

among geographically distinct populations of SWCB in their susceptibility to Cry1F insecticidal protein. However, all field-collected populations were more susceptible to Cry1F insecticidal protein than the MS lab colony (LC_{50} =7.88 μg/ml and EC_{50} = 0.163 μg/ml). The bioassay using growth inhibition is considered to be more sensitive in determining the susceptibility of the SWCB to Cry1F than using larval mortality because some larvae survive the high concentrations of Cry1F in the LC_{50} determination assay, but remain as first instar larvae at 14 DAT. In the diagnostic assay, these surviving larvae were counted as dead. But in the growth inhibition study, these surviving larvae were weighed and these weights were used to calculate the growth inhibition rate. Figure 1 shows the yearly comparisons of LC_{50} and EC_{50} values between and within field populations of SWCB tested. When the results of the field collected colonies are considered relative to the MS lab colony, no yearly trends are apparent.

The diagnostic concentration was estimated from the LC_{99} value of the MS lab colony, the least susceptible colony determined in feeding bioassay. Although a few larvae survived the diagnostic concentration at 14 after assay initiation, they remained the same size as first instars (the inoculated stage) and were considered dead because these larvae would not be able to survive in the natural environment. Thus, the mortality in diagnostic concentration for all 7-field populations was 100% (Table 2) because the LC_{99} value of MS lab colony was much higher than that of the 7 field populations tested in 2000.

This is the fifth year that the susceptibility of SWCB field populations to Cry1F insecticidal protein was bioassayed in this lab. **No significant changes in the susceptibility of SWCB to Cry1F were detected** when the data from this year were compared to that of the past four years (Figure 1).

In 2005, the sample protocol will be modified to increase the likelihood of collecting a large number of insects. In 2004, five populations of diapausing larvae were collected. A significant number of diapausing larvae died due to infection and physical damage. The surviving larvae pupated and emerged as adults in an unsynchronized manner, resulting in many unfertilized egg masses. As a consequence, the F1 generation

was had few individuals. It took additional time to increase the colony numbers high enough for the feeding bioassay. This caused a significant delay in completing the bioassay. To increase the chance for collecting SWCB females in 2005, we will send two teams simultaneously to two different locations in region 4 to collect the second and/or third flight females using blacklight traps. This will ensure that we will not miss the very short time of the peak for second or third flight females and allow us to collect an extra night or two at the same location if needed. Collection of diapausing larvae will be used as a last alternative. For region 2, Dr. Dan Mollenbeck will collect SWCB populations for our monitoring assay, preferably female adults from the same locations as in 2004. These modifications to the sampling protocol should help to ensure that adequate insects are collected for the 2005 growing season.

REFERENCES

Chippendale GM and Cassatt KL (1985) *Diatraea grandiosella,* in *Handbook of Insect Rearing, vol 2,* ed Singh P and Moore RF, Elsevier Science Publishers, Amsterdam, pp 257-264.

Table 1. 2003 SWCB collections.

Region*	State	County (Bt corn Penetration rate %)	Designation	Starting population (#)
4	Missouri	New Madrid (30-39)	Delta Center	188 DL
	Missouri	Stoddard (20-29)	Dexter	103 FA (FL_2)
	Kentucky	Caldwell	Princeton	73 FA (FL3)
	Illinois	Pulaski	Ullin	203 DL
2	Kansas	Stafford (>50%)	Great Bend	62 DL
	Kansas	Finney (10-19)	Garden City	153 DL
	Kansas	Gary	Cimarron	112 DL
Lab colony	Mississippi	Laboratory	MS Lab	>1,000 EM

* These two regions were selected for SWCB sample collection by ABSTC in March 2000.
DL=diapausing larvae; FL_2= second flight females; FL_3= third flight females.

Table 2. Susceptibility of SWCB neonate larvae to Cry1F *Bt* protein (14 days after treatment).

Collection Site	LC_{50} (95% CL) (μg/ml of diet)	LC_{95} (95% CL) (μg/ml of diet)	% Mortality* (diagnostic)
MO-New Madrid	1.85 (0.09-3.98)	19.28 (2.99-45.23)	100
MO-Stoddard	2.35 (0.27-6.01)	21.35 (3.80-63.07)	100
KY-Coldwell	2.14 (0.33-3.48)	18.62 (1.88-41.22)	100
IL-Ulin	1.90 (0.20-4.08)	14.47 (2.04-32.71)	100
KS-Stafford	1.20 (0.21-3.21)	25.8 (2.23-69.32)	100
KS-Finney	0.87 (0.17-3.62)	19.59 (3.48-54.53)	100
KS- Gary	2.57 (0.23-5.21)	24.54 (2,15-65.12)	100
Lab colony	7.88 (2.01-18.13)	68.65 (12.5-114.62)	100

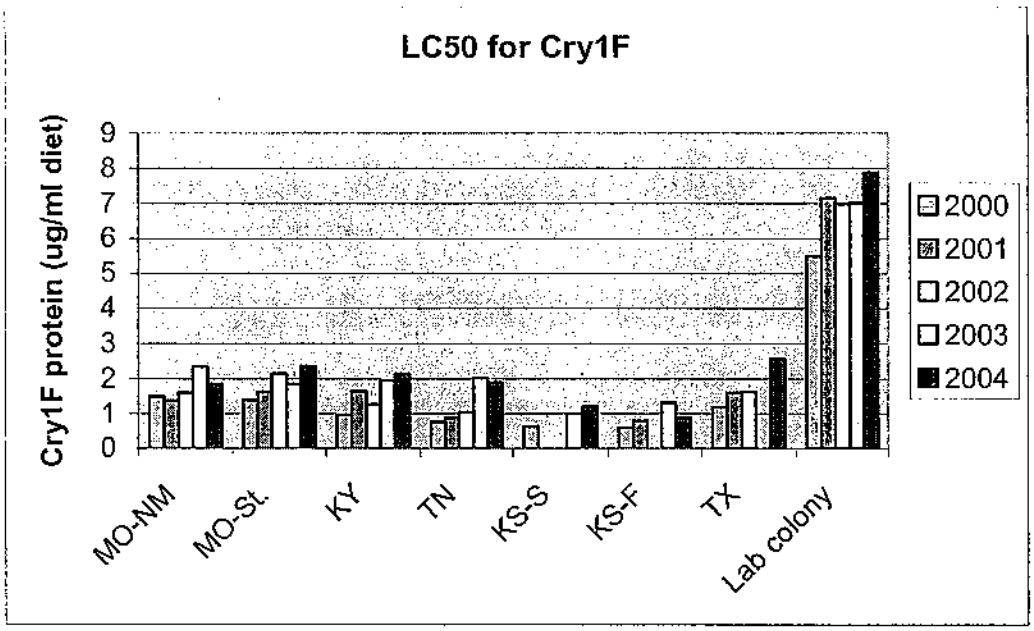
*: The diagnostic concentration was the LC_{99} of the MS laboratory population 14 DAT. 200 larvae from each field population were tested for the diagnostic concentration. The larvae were counted as dead if motionless when approached by a brush or if remaining as first instar larvae.

C. 87-2.57

Table 3. Growth inhibition of neonate larvae fed continuously on an artificial diet containing Cry1F protein (14 days after treatment).

Collection Site	EC_{50} (95% CL) (μg/ml of diet)	EC_{95} (95% CL) (μg/ml of diet)
MO-New Madrid	0.079 (0.018-0.197)	0.71 (0.24-1.98)
MO-Stoddard	0.098 (0.022-0.310)	0.81 (0.33-2.52)
KY-Coldwell	0.082 (0.019-0.230)	0.66 (0.14-1.03)
IL-Ulin	0.074 (0.031-0.189)	0.87 (0.22-2.61)
KS-Stafford	0.097 (0.029-0.264)	0.73 (0.10-2.78)
KS-Finney	0.104 (0.033-0.237)	0.93 (0.33-3.02)
KS-Gary	0.072 (0.016-0.203)	0.69 (0.20-2.03)
MS-Lab colony	0.163 (0.057-0.532)	2.94 (0.21-9.18)

0.072 - 0.104



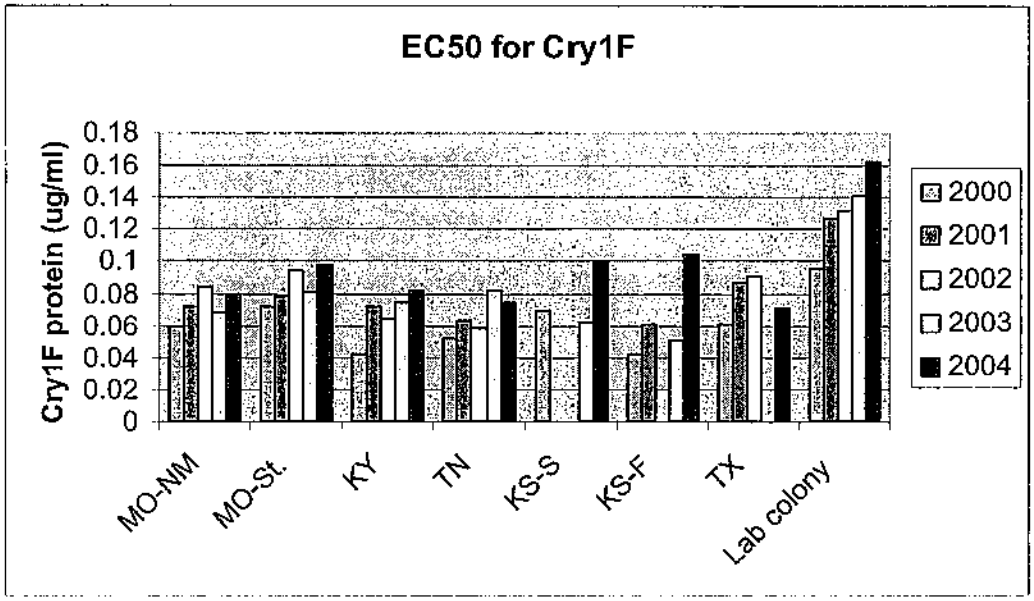


Figure 1. Yearly comparison of LC_{50} (top panel) and EC_{50} (bottom panel) of Cry1F protein in feeding bioassay between and within SWCB field populations and a lab colony.

Note: some data are not available for the following locations due to unsuccessful sample collection.

KS-St John: no data available for 2001 and 2002.
KS-Finney: no data available for 2001.
TX: no data available for 2003 and 2004. The 2004 data from Gary County (KS) were added here for comparison purpose.
TN: 2003 and 2004 data were replaced by the data from Ullin, IL.

Monitoring the Susceptibility of the Southwestern Corn Borer, *Diatraea grandiosella,* to *Bacillus thuringiensis* Cry1Ab toxin

2004 Data Summary

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(April 22, 2005)

INTRODUCTION

The effectiveness of the resistance management strategies is measured by monitoring field populations. This allows for early detection of resistance so that selected management strategies can be implemented before control failure occurs. To monitor the susceptibility of Southwestern corn borer, *Diatraea grandiosella*, a serious maize pest in the southern United States, to *Bt* toxin in transgenic maize, SWCB populations are collected from areas where the selection pressure for resistance is the greatest, i.e. the areas with the highest market penetration of *Bt* corn hybrids and /or the areas with the highest rate of insecticide application.

In this report, seven field populations of the SWCB were collected from two regions with high *Bt*-corn penetration rate and assayed along with a lab colony to determine their susceptibility (larval mortality, larval growth inhibition and response to diagnostic dose) to Cry1Ab toxin. The data were then compared with the previous years' data for any sign of changes in the susceptibility of SWCB to Cry1Ab toxin.

EXPERIMENT PROCEDURES

a. Field SWCB sample collection

The SWCB field samples were collected from the same/nearby locations with a high *Bt*-corn penetration rate and/or a history of insecticide application in refuge areas, as we did in the previous years. Dr. Dan Moellenbeck, DM Crop Research Group, under contract to ABSTC, collected three populations of SWCB from region 2 (Southwest

Kansas and the panhandle area of Oklahoma and Texas) and our laboratory collected four SWCB field populations from region 4 (MO, IL, TE, and KY).

In region 2, three populations of SWCB diapausing larvae were collected from Garden City (Finney County) (153 larvae), Cimarron (Gary County) (112 larvae) and Great Bend (Stafford County) (62 larvae) of KS by Dr. Moellenbeck. In region 4, a total of four SWCB populations were collected. One population of second flight adults (103 females at Dexter, MO) and one population of third flight adults (73 females at Princeton, KY) were collected using a blacklight trap over two or three consecutive nights. Insufficient adults were collected of second and third flights from the other two locations (<10 females/location), presumably due to low density or unfavorable weather conditions. As an alternative, we traveled in early October to the same areas and collected one population of diapausing larvae from Delta Center, MO (188 diapausing larvae) and one from Ullin, IL (203 diapausing larvae) (Table 1).

b. Rearing of SWCB

Larvae generated from the field-collected populations and from a Mississippi (MS) laboratory colony were maintained on a BioServe artificial diet in clear plastic cups (20 ml) using established laboratory procedures (Chippendale and Cassatt, 1985). Larvae were kept at 30^0C under 16 hr L : 8 hr D, whereas eggs, pupae, and adults were incubated at 25^0C under 13 hr L : 11 hr D.

c. Cry1Ab toxin

Cry1Ab toxin (Tryptic core, Lot # B8R176001, Batch 5, reference #7377600) was provided by Monsanto Company (Dr. Ty Vaughn), aliquoted and stored at -80^0C until use.

d. Susceptibility (LC_{50} and EC_{50}) of SWCB neonate larvae to Cry1Ab toxin

Larval mortality

A larval feeding bioassay was used to determine mortality. All bioassays were

performed in a 128-cavity tray using diet incorporated serial concentrations of Cry1Ab toxin. A minimum of 120 larvae were tested per concentration.

Cry1Ab toxin was diluted in 50 ml centrifuge tubes with each containing 1 ml of distilled water to make a serial dilution of desired concentrations. Each solution was incorporated into 40 ml of added artificial diet (kept at 55^0C in a water bath) by inverting and shaking the tube. One ml of diet containing the indicated concentration of Cry1Ab toxin was then pipetted into a 128-cavity tray (BIO BA 128 bioassay tray, C-D International, Ocean City, NJ). The trays were either assayed shortly after cool down to room temperature or kept at 4^0C for < 48 h before use. Newly hatched and actively moving larvae (<24 h) were transferred individually to each cavity containing 1 ml of diet with the indicated concentration of Cry1Ab toxin. Each concentration was replicated four times (32 larvae replicate^{-1}) using larvae from different batches of the F_1 or F_2 generations (if the number of F_1 larvae from field collected females were not large enough, F_2 larvae were used). Both F_2 and F_3 generation larvae were used for the bioassay if the field population was from diapausing larvae. Larval mortality was observed at 14 days after treatment (DAT). Larvae were considered dead if they did not move when they were probed using a camel hairbrush or if they remained at the first instar.

Seven consecutive concentrations of Cry1Ab toxin, including the control, were used for probit analyses to determine LC_{50} and LC_{95} values on 14 DAT. These concentrations produce larval mortality between 0 and 100%. Probit analyses were conducted, using SAS program (SAS Institute Inc., Cary, NC).

Larval growth inhibition

Six concentrations of Cry1Ab toxin were used in the feeding bioassay. Each concentration was replicated 4 times (32 larvae replicate^{-1}) as described above. The mean weight of newly hatch larvae (initial weight) was determined by weighing a group of 32 larvae and the mean weight of those larvae surviving at each tested concentration was determined at 14 DAT.

Regression analyses (log concentration vs. weight gain inhibition [%]) were used to determine the EC_{50} and EC_{95} values at 14 DAT. Weight gain inhibition represents the relative differences in weight gain of the treated larvae compared to that of the control larvae. The following equation was used to correct for initial weight: $I = \{(C - T) \div (C - B)\} \times 100\%$, where I = weight gain inhibition (%); C = mean weight of control larva (mg $larva^{-1}$); T = mean weight of surviving larvae from the treated diets (mg $larva^{-1}$); and B = mean initial larval weight (mg $larva^{-1}$).

Efficacy of the diagnostic concentration against the laboratory-adapted and field-collected populations of Diatraea grandiosella

The diagnostic concentration was estimated from the LC_{99} value at 14 DAT of the MS laboratory colony (obtained in 2000 from Frank Davis at the USDA-ARS laboratory in Stoneville, MS) because this population has been found to be the least susceptible to Cry1Ab protein of the populations tested. Seven field populations of SWCB were used to test the efficacy of the diagnostic concentration (5 μg/g diet for 14 DAT) in determining the susceptibility to Cry1Ab.

Two hundred newly hatched larvae from the F_2 or F_3 generation of each field population were transferred individually into the 128-cavity tray containing 1 ml of control diet or the diet with the diagnostic concentration of Cry1Ab toxin. At 14 DAT, larval mortality was recorded.

RESULTS AND DISCUSSIONS

Bioassay results of the susceptibility of neonate SWCB larvae to Cry1Ab toxin are summarized in Tables 2 and 3.

Two bioassay procedures (larval mortality vs. growth inhibition) were employed to investigate the susceptibility of SWCB to Cry1Ab. Based on the LC_{50} (Table 2) and EC_{50} (Table 3), significant variation [LC_{50} from 0.10 to 0.28 μg/ml diet for 14 DAT (Table 2), EC_{50} from 3.33 to 6.11 ng/ml diet for 14 DAT (Table 3)] was observed among

geographically distinct populations of SWCB in their susceptibility to Cry1Ab toxin. However, all field-collected populations were more susceptible to Cry1Ab toxin than the MS-lab colony (LC_{50} =0.55 µg/ml and EC_{50} = 11.32 ng/ml). The bioassay using growth inhibition is considered to be more sensitive in determining the susceptibility of the SWCB to Cry1Ab than using larval mortality because some larvae survive high concentrations of Cry1Ab in the LC_{50} determination assay, but remain as first instar larvae. In the diagnostic assay, these surviving larvae were counted as dead. However, in the growth inhibition study, these surviving larvae were weighed and these weights were used to calculate the growth inhibition rate. Figure 1 shows the yearly comparisons of LC_{50} and EC_{50} between and within field populations of SWCB tested. When the results of the field collected colonies are considered relative to the MS lab colony, no yearly trends are apparent.

The diagnostic concentration was estimated from the LC_{99} value of the MS lab colony, the least susceptible colony determined in feeding bioassay. Although a few larvae survived the diagnostic dose at 14 days after initiating the assay, they remained at the size of first instar (the inoculated stage) and were considered dead because these larvae would not be able to survive in the natural environment. Thus, the mortality in diagnostic concentration of Cry1Ab toxin for all 7-field populations was 100% (Table 2) because the LC_{99} value of MS lab colony was much higher than that of the 7 field populations tested in 2000.

This is the fifth year that the susceptibility of SWCB to Cry1Ab protein was bioassayed in this lab. **No significant changes in the susceptibility of SWCB to Cry1Ab were detected** when the data from this year were compared to that of the past four years (Figure 1).

For 2005 collections, the sample protocol will be modified to increase the likelihood of collecting a large number of insects. In 2004, five populations of diapausing larvae were collected. A significant number of diapausing larvae died due to infection and physical damage. The surviving larvae pupated and emerged as adults in an unsynchronized manner, resulting in many unfertilized egg masses. As a consequence,

the F1 generation had very few individuals. It took additional time to increase the colony numbers high enough for the feeding bioassay. This caused a significant delay in completing the feeding bioassay. To increase the chances for collecting SWCB females in 2005, we will send two teams simultaneously to two different locations in region 4 to collect second and/or third flight females in blacklight traps. This will ensure that we will not miss the very short time for peak flights and allow us to collect an extra night or two at the same location if needed. Collection of diapausing larvae will be used as a last alternative. For region 2, Dr. Dan Moellenbeck will collect SWCB populations for our monitoring assay, preferably as female adults from the same locations as 2004. These modifications to the sample protocol should help to ensure that adequate insects are collected for the 2005 growing season.

REFERENCES

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2	Kansas	Stafford (>50)	Great Bend	62 DL
	Kansas	Finney (10-19)	Garden City	153 DL
	Kansas	Gary	Cimarron	112 DL
Lab colony	Mississippi	Laboratory	MS Lab	>1,000 EM

* These two regions were selected for SWCB sample collection by ABSTC in March 2000.
DL=diapausing larvae; FA=female adults; FL_2= second flight females; FL_3= third flight females.

Table 2. Susceptibility of SWCB neonate larvae to the Cry1Ab *Bt* toxin (14 days after treatment).

Collection Site	LC_{50} (95% CL) (µg/ml of diet)	LC_{95} (95% CL) (µg/ml of diet)	% Mortality* (diagnostic)
MO-New Madrid	0.11 (0.04-0.19)	0.38 (0.10-0.78)	100
MO-Stoddard	0.13 (0.05-0.31)	0.42 (0.12-1.14)	100
KY-Coldwell	0.10 (0.03-0.19)	0.62 (0.22-1.52)	100
IL-Ullin	0.17 (0.06-0.25)	0.55 (0.21-0.99)	100
KS-Stafford	0.11 (0.03-0.28)	0.58 (0.20-1.14)	100
KS-Finney	0.28 (0.11-0.44)	0.49 (0.13-1.07)	100
KS-Gary	0.22 (0.02-0.51)	0.61 (0.17-1.35)	100
Lab colony	0.55 (0.27-0.89)	2.04 (0.90-5.31)	100

* The LC_{99} of the Cry1Ab Bt toxin was the LC_{99} value at 14 DAT of the MS laboratory population. Any larvae that were alive, but remained as first instar, were counted as dead.

0.10-0.28

Table 3. Growth inhibition of neonate larvae fed continuously on an artificial diet containing Cry1Ab *Bt* toxin (14 days after treatment).

Collection Site	EC50 (95% CL) (ng/ml of diet)	EC95 (95% CL) (ng/ml of diet)
MO-New Madrid	3.33 (0.97- 8.68)	49.80 (18.7-134.0)
MO-Stoddard	6.02 (1.40-13.21)	55.17 (10.7-117.2)
KY-Coldwell	5.51 (0.89-15.51)	39.04 (15.3- 94.6)
IL-Ulin	4.09 (1.02-12.71)	33.17 (9.4-102.2)
KS-Stafford	6.07 (2.28-17.18)	48.15 (11.2- 98.2)
KS-Finney	5.29 (0.98-21.01)	37.98 (7.3- 67.4)
KS-Gary	6.11 (1.27-19.21)	51.22 (9.5- 87.4)
MS-Lab colony	11.32 (1.31-48.13)	103.04 (24.3-178.2)

3.33 - 6.11

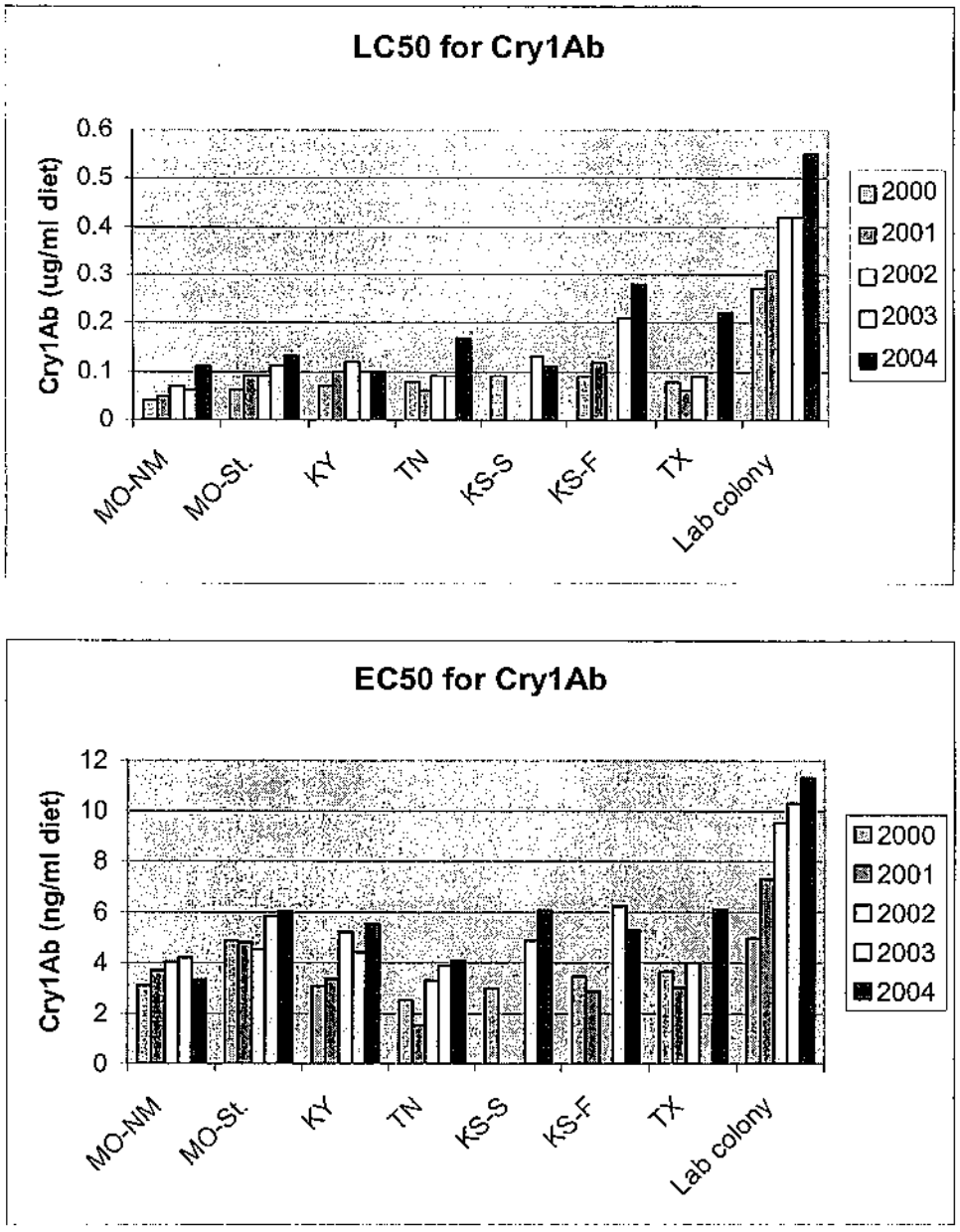

Figure 1. Yearly comparison of LC_{50} (top panel) and EC_{50} (bottom panel) of Cry1Ab toxin in feeding bioassay between and within SWCB field populations and a lab colony.

Note: some data are not available for the following locations due to unsuccessful sample collection.

KS-Stoddard: no data available for 2001 and 2002.

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TN: 2003 and 2004 data were replaced by the data from Ullin, IL.